Journal of Chromatography, 233 (1982) 269—278
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1427

SENSITIVE ASSAY FOR THE TRICYCLIC ANTIDEPRESSANT Ro 11-2465 IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

P. HAEFELFINGER

Biological Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd., CH-4002 Basle (Switzerland)

(Received May 4th, 1982)

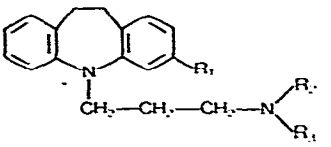
SUMMARY

A high-performance liquid chromatographic assay, suitable for pharmacokinetic studies, has been developed for the new tricyclic antidepressant Ro 11-2465, at present under clinical investigation. For concentrations above 0.5 ng/ml, the method involves a simple extraction at basic pH with an organic solvent followed by direct chromatography of this extract on a silica gel column using fluorescence detection. For concentrations below 0.5 ng/ml, an extensive clean-up procedure is required. In both procedures, however, evaporation of the extract and reconstitution of the residue is avoided. The detection limit, using 1 ml of plasma, is about 0.1 ng/ml. This sensitivity is sufficient for following single-dose kinetics of Ro 11-2465 in man.

INTRODUCTION

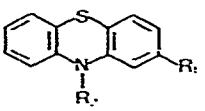
Tricyclic drugs such as imipramine and clomipramine (Fig. 1) are widely used as antidepressants. Pharmacokinetic and pharmacological studies have prompted the development of several analytical procedures for monitoring the plasma and urine levels of these drugs. However, the concentrations of these drugs in biological fluids are extremely low and the development of a suitable method is difficult. Many papers using thin-layer chromatography, gas chromatography, mass spectrometry and high-performance liquid chromatography (HPLC) have been published [1], but not all of these procedures are sensitive enough for single-dose pharmacokinetics. During the last few years, the use of HPLC has become increasingly important for these compounds [2]. Imipramine was given orally to volunteers in doses of 25 mg and plasma levels of less than 10 ng/ml were reported [3]. In a pharmacokinetic study with

0378-4347/82/0000—0000/$02.75



	R_1	R_2	R_3
Imipramine	-H	$-CH_3$	$-CH_3$
Clomipramine	-Cl	$-CH_3$	$-CH_3$
Ro 11-2465 *)	-CN	$-CH_3$	$-CH_3$
N-desmethyl compound of Ro 11-2465	-CN	$-CH_3$	-H
N-bisdesmethyl compound of Ro 11-2465	-CN	-H	-H

Internal standards



	R_1	R_2
Promazine	-H	$-CH_2-CH_2-CH_2-N(CH_3)_2$
Thioridazine	$-SCH_3$	$-CH_2-CH_2-$(N-CH_3-piperidine)

Fig. 1. Chemical formulae of the relevant compounds. *Ro 11-2465 = 5-[3-(dimethylamino)-propyl]-10,11-dihydro-5H-dibenz[*b*,*f*]azepine-3-carbonitrile (USP 4'138'482).

clomipramine, the dose was 50 mg orally and the plasma levels in healthy subjects were less than 30 ng/ml [4].

The imipramine derivative Ro 11-2465 (Fig. 1) is a potent and selective inhibitor of serotonin uptake and is currently under development as an antidepressant drug. Due to its potency, the highest dose given to volunteers for pharmacokinetic studies was 4 mg, orally. Since this compound is similar to imipramine and clomipramine, the expected plasma levels are below about 5 ng/ml. Preliminary experiments with thin-layer and gas chromatography were not successful. An HPLC procedure using UV detection has been reported, which is capable of determining nanogram quantities of tricyclic drugs [5]. Highly sensitive methods for imipramine using fluorescence detection have been reported [6, 7] and our own laboratory has had good experience with this detection method [8, 9]. Considerable preliminary work was necessary in order to establish the optimal HPLC and fluorescence conditions.

Losses of tricyclic drugs on glass surfaces is a well-known phenomenon [10, 11], and for this reason evaporation of extracts to dryness, followed by reconstitution of the residue, should be avoided whenever possible. Therefore, a procedure was developed in which the extracts could be chromatographed directly. Two different sample preparation procedures were used: for concentrations above 0.5 ng/ml plasma a single extraction from biological material and chromatography of the extract was suitable; for concentrations below 0.5

ng/ml, an extended sample preparation procedure with back-extraction was necessary.

EXPERIMENTAL

Materials

NaOH 1 *N*, ammonia 33% (B.P. 1953, stored at —20°C), 2% H_3PO_4, methanol (Uvasol), diisopropyl ether p.a. (freshly distilled and stored in the dark), and *n*-hexane p.a. were supplied by E. Merck, Darmstadt, G.F.R.

For the extraction, the following mixtures were used: 2.5% methanol in diisopropyl ether, and 2% isoamyl alcohol in *n*-hexane.

Ro 11-2465 was first synthesized by Dr. Dostert. The metabolites were synthesized by Dr. Joos and Dr. Hunkeler, Chemical Department of Roche, Basle, Switzerland. The internal standard promazine is commercially available and thioridazine was supplied by Sandoz, Basle, Switzerland.

Equipment and chromatography

The liquid chromatograph consisted of an Altex (Berkeley, CA, U.S.A.) pump Model 110, a Rheodyne (Berkeley, CA, U.S.A.) 71-25 high-pressure sample valve with a 300-μl loop, or a Kontron (Zürich, Switzerland) automatic sample injector MSI 660 with a 200-μl loop.

A stainless-steel column (250 × 3.2 mm), filled with LiChrosorb Si 60 (Merck), 5 μm particle size, was used. A Perkin-Elmer (Norwalk, CT, U.S.A.) fluorimeter 650-10 LC was operated with the following instrument settings: excitation wavelength 280 nm, slit 15 nm, emission wavelength 410 nm, slit 20 nm, sensitivity range 1, fine 5, PM gain norm, response slow, mode norm. To reduce the amplitude of the short-term noise of the baseline, an additional output filter with a time constant of 2 or 4 sec was used.

To control the chromatographic system, it was advantageous to connect a UV detector in series with the fluorimeter. A Uvikon 725 (Kontron) detector with a deuterium lamp was used, wavelength 242 nm, range 0.01, time constant 3. The chromatograms were obtained on a W + W recorder, Model 1200 (Kontron), UV channel 10 mV, fluorescence channel 1 mV, chart speed 5 mm/min.

The mobile phase consisted of 0.1 ml of ammonia 33%, mixed with 6 ml of methanol and adjusted to 100 ml with distilled diisopropyl ether. Using a flow-rate of 2 ml/min, the pressure was about 200—300 bar. The retention times were approx. 4 min for Ro 11-2465 and 5—6 min for the internal standard — promazine or thioridazine.

Preparation of the glassware

The adsorption effects of Ro 11-2465 on glass surfaces could be considerably reduced by treating all the glassware with alkaline methanol (0.5 ml of 14 *N* potassium hydroxide in 100 ml of methanol), followed by rinsing with pure methanol and air drying. Polypropylene tubes, rinsed with methanol and air dried, were also suitable for the assay.

Standards

Amber glass or polypropylene volumetric flasks were used to prepare the standard solutions.

The stock solution contained 5 mg of Ro 11-2465 as hydrochloride in 10 ml of methanol. Further dilutions in methanol covering the concentration range 25 μg/ml down to 15 ng/ml were obtained, starting from the stock solution. These methanolic solutions could be stored for up to three months at −20° C.

Calibration solutions for the chromatographic system and for recovery studies were prepared as follows: To 200 μl of the methanolic solutions were added 50 μl of methanol (which may contain the internal standard, see below). The volume was adjusted to 10 ml with diisopropyl ether. The concentration range was 0.625—20 ng/ml.

Plasma and urine standards were prepared by dilution of 200 μl of the appropriate methanolic standard solution with drug-free plasma or urine to 25 ml. The concentration range for plasma was 4—0.125 ng/ml and for urine 200—6.25 ng/ml. These standards were stored in polypropylene tubes in aliquots of 2.5 ml at −20° C.

Sample preparation

Plasma or urine (1 ml) was mixed with sodium hydroxide solution (1 *N*, 0.2 ml) and 2.5% methanol in diisopropyl ether (1 ml) in a polypropylene tube or a glass tube treated as described above. The tubes were rotated for 5 min (rotary tube mixer, REAX II; Heidolph Elektro AG, Keilheim, G.F.R.) and then centrifuged at 1200 *g* for 5 min. A portion of the organic extract (200 μl) was chromatographed.

Along with the unknown samples, 4—5 plasma standards were analysed, covering the expected concentration range.

This procedure was suitable for the concentration range 0.5—20 ng/ml. For higher concentrations, which have been observed in urine, the volume of the extraction mixture was increased to 2 ml or more. For plasma concentrations below 0.5 ng/ml, an extended sample preparation procedure was used as follows. Plasma (1 ml) was mixed with sodium hydroxide (1 *N*, 0.2 ml). This mixture was extracted twice with 2% isoamyl alcohol in *n*-hexane (5 ml). The combined organic phase was extracted with 2% H_3PO_4 (1 ml). After discarding the organic phase, the aqueous phase was basified with sodium hydroxide (1 *N*, 1 ml) and extracted with 2.5% methanol in diisopropyl ether (0.5 ml). A portion of this extract (200 μl) was chromatographed. This extraction procedure is described in further detail elsewhere [12].

Calibration and calculation

The plasma or urine standards analysed along with the unknown samples were used for calculation of the unknown concentrations. A calibration curve was obtained by calculation of the least-square regression of the peak heights of the plasma or urine standards versus the concentrations of Ro 11-2465. Using these curves the concentrations of the drug in the unknown samples were calculated.

Internal standard

Thioridazine (Fig. 1) was tried as internal standard. The substance dissolved in sodium hydroxide (1 *N*, 0.2 ml) was added to the samples. Due to adsorption effects or instability in alkaline solution, poor results were obtained. Preliminary experiments showed that promazine was more suitable. It could be added to the samples as an aqueous solution (50 or 100 μl). The quantity of promazine had to be chosen such that 200 μl of the extract (which is chromatographed) contained 50 ng. It is not yet known whether or not promazine fulfils the conditions for the internal standard technique [13].

RESULTS

Characteristics of the method

Selectivity. Ro 11-2465 is well separated from endogenous plasma interferences under the chromatographic conditions described (Fig. 2). In human urine and in plasma of rats treated with Ro 11-2465 two substances with retention times of about 15—17 and 21 min, respectively, were observed. The substance which elutes at 15 min is probably the N-bisdesmethyl, and that at 20 min the N-desmethyl compound of Ro 11-2465 (Figs. 1 and 3). Due to the very low quantities present in biological fluids, a definite identification of these compounds has not yet been possible. Chromatograms of standards and of extracts of human plasma spiked with Ro 11-2465 are shown in Fig. 2.

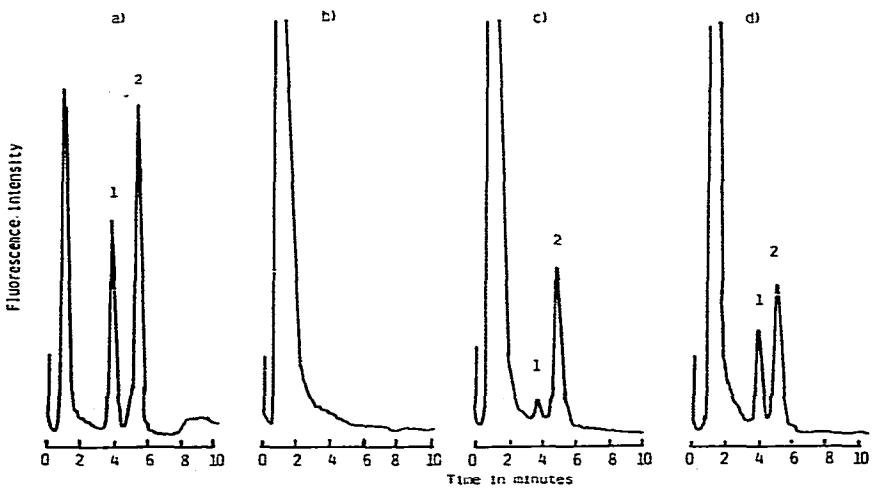


Fig. 2. (a) Chromatogram of 1 ng of Ro 11-2465 (hydrochloride) and 40 ng of thioridazine, injected in 200 μl of 2.5% methanol—diisopropyl ether. (b) Chromatogram of the extract of human blank plasma: 200 μl injected, single extraction. (c) Chromatogram of the extract of human plasma, spiked with 0.5 ng/ml Ro 11-2465 (hydrochloride) and 100 ng of thioridazine: 200 μl injected, single extraction. (d) Chromatogram of the extract of human plasma, spiked with 2 ng/ml Ro 11-2465 (hydrochloride) and 100 ng of thioridazine: 200 μl injected, single extraction. Peaks: 1 = Ro 11-2465; 2 = thioridazine. Chromatographic conditions: column 250 mm × 3.2 mm, LiChrosorb Si 60, 5 μm; mobile phase: 0.1 ml ammonia 33% + 6 ml methanol, with diisopropyl ether to 100 ml, flow-rate 2 ml/min; fluorescence detection at 280 nm/410 nm.

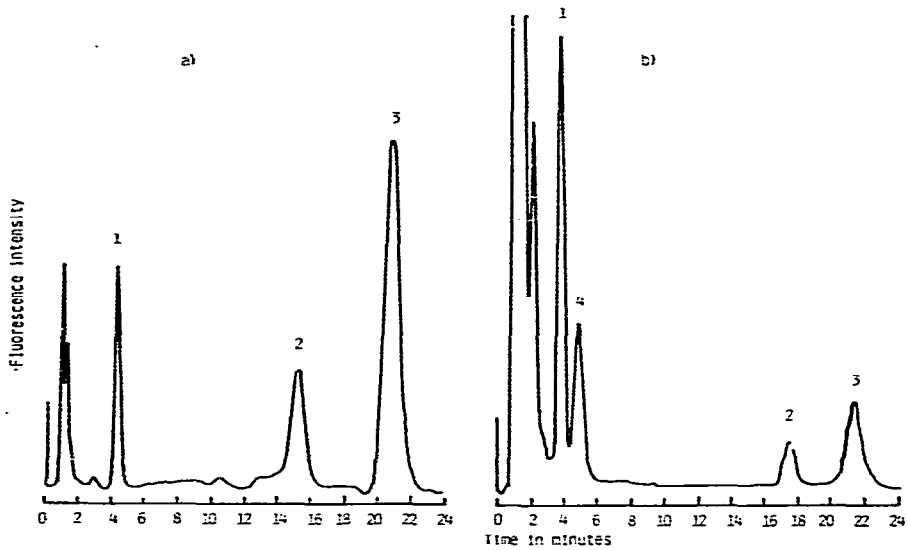


Fig. 3. (a) Chromatogram of rat plasma, collected 90 min after an oral dose of 50 mg/kg Ro 11-2465 (hydrochloride). Single extraction, 200 μl injected. (b) Chromatogram of the urine of a volunteer, collected 14—24 h following a single oral dose of 4.48 mg of Ro 11-2465 (hydrochloride); 0.5 ml of urine were mixed with 0.2 ml of 1 *N* NaOH, containing 2.5 μg of thioridazine, extracted with 5 ml of 2.5% methanol—diisopropyl ether and 200 μl of the extract were injected. Peaks: 1 = Ro 11-2465; 2 = probably the N-bisdesmethyl compound of Ro 11-2465; 3 = probably the N-desmethyl compound of Ro 11-2465; 4 = thioridazine. Chromatographic conditions as in Fig. 2.

Linearity. The response of the fluorescence detector was linear in the range 0.05—4 ng of Ro 11-2465 injected from a 200-μl extraction mixture. For most determinations, the instrument settings were chosen such that 2 ng of Ro 11-2465 (hydrochloride) gave full-scale deflection. Since the concentration of Ro 11-2465 in biological fluids is low, the linearity above 4 ng injected was not tested.

Recovery. The extraction yield (recovery) of Ro 11-2465 was above 95% for both the simple and extended sample preparation procedures.

Accuracy. The accuracy of a method may be defined as the difference between the mean value of replicate assays of the same sample and the true value. Spiked plasma samples were prepared for this purpose and the results are summarized in Table I.

Precision. The precision of a method is represented by the relative standard deviation of the mean of replicate assays of the same sample. The precision was estimated in plasma and urine by analysing the same unknown samples on different days. In this case, for plasma, a classification into two ranges was possible [14]. For concentrations from 0.8 to 4 ng/ml plasma, the relative standard deviation of the method was 6%; below 0.8 ng/ml it was 13%. These values were obtained with clinical samples, not with spiked plasma. In urine, the relative standard deviation of the method was about 9% in the concentration range 5—100 ng/ml. Accuracy and precision data from spiked samples are given in Table I and precision from clinical trials in Table II.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY OF Ro 11-2465 IN SPIKED HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found (ng/ml)	*n* replicates	Percentage of the added amount	Coefficient of variation (%)
0.30	0.33	11	110	17
0.60	0.56	14	93	12
0.90	0.89	8	99	5.6
1.20	1.20	15	100	6.3
2.40	2.37	15	99	5.5

TABLE II

PRECISION DATA FOR Ro 11-2465 FROM THE CLINICAL TRIALS

	Concentration range (ng/ml)		
	<0.8	0.8—4	5—100
Plasma	13%	6%	
Urine			9%

Detection limit. The detection limit for Ro 11-2465, defined as the amount injected giving rise to a signal-to-noise ratio of 3:1, was 0.05 ng (from a 200-μl sample). Using 1 ml of plasma and 1 ml of extraction mixture, the detection limit for the simple sample preparation procedure was 0.3 ng/ml. Using the extended clean-up procedure, a detection limit of about 0.1 ng/ml, or even lower, could be achieved for optimal instrument settings. In urine the detection limit was about 5 ng/ml; due to endogenous interferences, it would be difficult to improve this value.

Stability. Plasma samples spiked with Ro 11-2465 were stored for one year at —20°C. In the concentration range 1—5 ng/ml, no measurable degradation of the compound could be observed.

Analysis of plasma and urine samples

The method described was used to determine plasma and urine levels of Ro 11-2465 from single-dose pharmacokinetic studies. The concentrations of Ro 11-2465 in the plasma and urine of a volunteer following a single oral dose of 4.48 mg of the hydrochloride, corresponding to 4 mg of the base, are summarized in Tables III and IV, respectively. Plasma levels were detectable up to 72 h post administration of the drug, and sufficient data were obtained to allow the calculation of the pharmacokinetic parameters.

As mentioned above, two peaks were observed in the chromatograms from rat plasma after oral administration of 50 mg/kg Ro 11-2465; these probably correspond to the N-desmethyl and N-bisdesmethyl compounds of Ro 11-2465

TABLE III

PLASMA LEVELS OF A VOLUNTEER (C.C.) FOLLOWING A SINGLE ORAL DOSE OF 4.48 mg OF THE HYDROCHLORIDE OF Ro 11-2465 AS CAPSULES

Time (h) after administration	Ro 11-2465 (as hydrochloride) (ng/ml)
0.25	<0.1
1	0.18
2	1.05
3	1.79
5	2.52
8	2.11
12	1.96
24	1.30
36	0.87
48	0.57
60	0.34
72	0.12

TABLE IV

DETERMINATION OF Ro 11-2465 IN THE URINE OF A VOLUNTEER (C.C.) FOLLOWING A SINGLE ORAL DOSE OF 4.48 mg OF THE HYDROCHLORIDE AS CAPSULES

Collection period post administration (h)	Quantity of urine (ml)	Concentration of Ro 11-2465 (hydrochloride) (ng/ml)	Amount of Ro 11-2465 (hydrochloride) excreted (ng/ml)
0—2	97	<5	—
2—4	113	35.9	4.1
4—6	193	129	24.9
6—8	115	54.9	6.3
8—10	87	87.3	7.6
10—12	107	141	15.1
12—14	125	6	0.8
14—24	480	26.6	12.8
24—36	570	25.0	14.3
36—48	470	38.6	18.1
48—72	770	22.0	16.9
72—96	1295	8.6	11.1
96—120	790	<5	—

(Fig. 3a). In human urine, similar peaks were found (Fig. 3b). In human plasma, however, these metabolites were not detected after a single dose of 4.48 mg of the hydrochloride, the concentrations being below the detection limit. Possible accumulation of these metabolites could be ascertained from multiple-dose studies.

DISCUSSION

Imipramine and desimipramine have been determined in plasma by HPLC with fluorescence detection [6]. Using 2 ml of plasma, a detection limit of about 0.5 ng/ml was attained. The procedure described here has considerably higher sensitivity; with 1 ml of plasma, 0.1 ng/ml Ro 11-2465 was detectable. With the extended sample preparation procedure this limit could be lowered to 0.05 ng/ml, using 2 ml of plasma.

The application of normal-phase HPLC has several advantages. The life-time of a normal-phase column is, generally, much longer than that of a reversed-phase column: more than 1000 plasma extracts could be analysed with the same column. Furthermore, it was possible, without evaporation and back-extraction, to determine concentrations of 0.3—0.5 ng/ml of plasma following a single extraction.

The reproducibility of this procedure is better than that described in published methods [2, 4, 6, 7], especially in the concentration range 1 ng/ml and less.

The development of the present assay involved extensive experiments. Poor results obtained initially were probably due to the irreversible adsorption of Ro 11-2465 to glass and plastic surfaces. This effect is well known for other tricyclic drugs [10, 11] and may differ from one vessel to another. Stock solutions in diisopropyl ether in untreated volumetric glass flasks showed no loss for several weeks, whereas in other identical flasks large amounts of Ro 11-2465 disappeared within a few hours. Treatment of the glassware as described above reduced this effect considerably. Good results were also obtained with polypropylene tubes.

The adsorption effect of the internal standard, thioridazine, could not be reduced by using alkali-treated glassware or polypropylene tubes. For other tricyclic compounds, this phenomenon could be reduced in acidic solutions [10], but we had no success with this approach. Preliminary experiments with promazine gave promising results, although with a similar substance, chlorpromazine, irreproducible extraction yields have been reported [11].

The reproducibility of the assay for Ro 11-2465, without using an internal standard technique, is good compared to the values published for other tricyclic compounds. It is questionable whether promazine or any other internal standard would improve the precision of the method [13], and therefore little time was spent looking for other internal standards.

The high sensitivity and the good reproducibility of this assay are strongly dependent on the fluorimeter; proper calibration of the system and the quality of the xenon lamp are of fundamental importance. Furthermore, the purity of mobile phase, especially diisopropyl ether*, can influence the baseline noise of the chromatogram. Selection of suitable reagents is therefore important. Unexpected fluorescence peaks, usually a result of problems either with the fluorimeter or the chromatographic system, are sometimes encountered. The

*Recent experiments have shown that by slight modification of the mobile phase diisopropyl ether can be replaced by *tert.*-butyl methyl ether. The baseline noise is considerably reduced with this solvent.

origin of such peaks may be readily identified using a UV detector in series with the fluorimeter. Changes in the quality of the solvents can be observed by comparing the baseline drift of both the UV and fluorescence record. This assay has been developed for single-dose kinetics of Ro 11-2465 in man following an oral dose of 4.48 mg of the hydrochloride. The sensitivity needed has been achieved, and it was possible to calculate pharmacokinetic parameters.

It is likely that this procedure can be modified for the assay of other tricyclic drugs in plasma or urine.

ACKNOWLEDGEMENTS

The author is grateful to Miss R. Hartenbach and Mr. B. Hess for their conscientious technical assistance and Dr. D. Dell for correction of the manuscript.

REFERENCES

1 R.N. Gupta and G. Molnar, Biopharm. Drug Dispos., 1 (1980) 259—278.
2 J. Godbillon and S. Gauron, J. Chromatogr., 204 (1981) 303.
3 H. d'A. Heck, N.W. Flynn, S.E. Buttrill, Jr., R.L. Dyer and M. Anbar, Biomed. Mass Spectrom., 5 (1978) 250.
4 J.-P. Dubois, W. Küng, W. Theobald and B. Wirz, Clin. Chem., 22 (1976) 892.
5 D.R.A. Uges and P. Bouma, Pharm. Weekbl. Sci. Ed., 1 (1979) 45.
6 P.A. Reece and R. Zacest, J. Chromatogr., 163 (1979) 310.
7 T.A. Sutfin and W.J. Jusko, J. Pharm. Sci., 68 (1979) 703.
8 P. Haefelfinger, J. Chromatogr., 221 (1980) 327.
9 P. Haefelfinger, Chromatographia, 14 (1981) 212.
10 G. Carnis, J. Godbillon and J.P. Metayer, Clin. Chem., 22 (1976) 817.
11 J. Fekete, P. Del Castilho and J.C. Kraak, J. Chromatogr., 204 (1981) 319.
12 P. Haefelfinger, J. Chromatogr. Sci., 17 (1979) 345.
13 P. Haefelfinger, J. Chromatogr., 213 (1981) 73.
14 P. Haefelfinger and M. Wall, Z. Anal. Chem., 307 (1981) 271.